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IMMUNOADJUVANT ACTIVITIES OF CELL WALLS AND THEIR WATER-SOLUBLE FRACTIONS PREPARED FROM VARIOUS GRAM-POSITIVE BACTERIA[1]

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SUMMARY The cell walls from all 21 species of gram-positive bacteria examined, except lysozyme-susceptible *Micrococcus lysodeikticus* (NCTC 2665) and lysozyme-resistant *Staphylococcus epidermidis* (ATCC 155), were found to be definitely adjuvant-active in both stimulation of increased serum antibody levels and induction of delayed-type hypersensitivity to ovalbumin when administered to guinea-pigs as water-in-oil emulsions. Using various cell wall lytic enzymes, the immunoadjuvant principles were solubilized with full retention of the adjuvant activities from walls of *Staphylococcus aureus* (Copenhagen), *Streptococcus pyogenes* (group A, type 6; S43/100), *Streptococcus salivarius* (IFO 3350), *Streptococcus faecalis* (IFO 12580), *Streptococcus mutans* (BHT), *Lactobacillus plantarum* (ATCC 8014), *Bacillus megaterium* (IFO 12068), *Corynebacterium diphtheriae* (Park-Williams No. 8), *Mycobacterium smegmatis*, and *Actinomyces viscosus* (ATCC 15987). Evidence was obtained that the non-peptidoglycan portion of the cell walls is not essential for manifestation of immunoadjuvancy.

INTRODUCTION

Following the pioneer studies of Schwab et al. on the cell walls of Group A *Streptococcus pyogenes* (Schwab, Cromartie and Robertson, 1959; Cromartie, Schwab and Craddock, 1960), a number of investigations have revealed that bacterial cell walls possess a variety of characteristic biological, pathological and immunological activities (Narita and Kotani, 1972). This would seem to be a natural consequence of the fact that bacterial cell walls, together with the capsule, form the outermost layer which is in contact with environmental factors. Moreover, the cell walls of parasitic microbes probably contain compounds which enable these organisms to survive in the host

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and evoke a variety of responses in the cells and tissues, including the immune responses.

The peptide-containing wax D, a peptidoglycolipid component of mycobacterial cell walls is a well-known immunological adjuvant, which stimulates increased serum antibody levels and also induces delayed-type hypersensitivity to a protein antigen (Lederer, 1971). It has also been shown that free, chloroform-soluble lipid and firmly bound lipid isolated from *Corynebacterium diphtheriae* cell walls exhibit significant adjuvant activity in the stimulation of either humoral or cellular immune responses to ovalbumin in guinea-pigs (Kotani et al., 1963b). In recent studies, Lederer and his associates and others (Adam et al., 1972; Chedid et al., 1972; Migliore and Jollès, 1972; Hiu, 1972, Adam et al., 1973; Migliore-Samour and Jollès, 1973) isolated a water-soluble principle with adjuvant activity from the cell walls or whole cells of *Mycobacterium* and phylogenetically related bacteria using lysozyme, autolytic processes or chemical means.

All the immunoadjuvants hitherto reported to be active in induction of delayed-type hypersensitivity and in stimulation of increased circulating antibody levels seem to contain a common peptidoglycan or its water-soluble derivative, which consists of the characteristic amino sugars and amino acids of bacterial cell walls. If this is the case, it seems likely that bacterial cell walls in general have definite immunological adjuvant activities. The purposes of the present investigation were: (1) To study the immunoadjuvant activities of cell walls from a variety of gram-positive bacteria, differing in detailed chemical composition and structure, but similar in the fundamental molecular pattern of their peptidoglycan portions, and (2) to solubilize and characterize the factor or structure responsible for the immunoadjuvant activities of these cell walls. It was hoped that by elucidating the relationship between the chemical structure and immunoadjuvant activity it would be possible to develope an adjuvant, which was potent in induction of cellular immunity and which caused no harmful side effects.

MATERIALS AND METHODS

1. *Cell wall preparations*

To prepare cell walls, cells were disrupted in a Braun Mechanical Cell Homogenizer (Model MSK) or a Kubota Sonic Oscillator (10 Kc/sec, Model KMS–100) and then subjected to differential centrifugation and treatment with crystalline trypsin or Pronase (Kaken Chemicals Co.) as described previously (Kotani et al., 1959a, 1959b; Suginaka et al., 1967; Matsuda, Kotani and Kato, 1968; Kotani et al., 1970; Hamada et al., 1971). The purity of isolated cell walls was checked by microscopic examination of gram-stained smears, by electron microscopy or by ultraviolet spectroscopy for detection of contaminating proteins and nucleic acids. The cell walls of (i) *Clostridium botulihum* (190L), (ii) *Nocardia asteroides* (131), and (iii) *Streptomyces fradiae* (IAM 0093) and *Streptomyces lavendurae* (IAM 0023) were gifts from Dr. K. Takumi, Department of Food Microbiology, Tokushima University School of Medicine (Takumi and Kawata, 1970, 1971), Dr. I. Azuma, Department of Internal Medicine, Osaka University Medical School (Azuma et al., 1973) and Dr. T. Nakamura, Central Research Laboratory, Takara Brewery Co. (Nakamura, Tamura and Arima, 1967), respectively. The cell walls of (iv) *Streptococcus salivarius* (HHT and IFO 3350), *Streptococcus mutans* (KIR), *Streptococcus bovis* (IFO 12058), *Streptococcus faecalis* (IFO 12580), *Streptococcus faecium* (IFO 3181), *Streptococcus lactis* (IFO 12546) and *Streptococcus thermophilus* (IFO 3535) were generously supplied by Drs. S. Kawata and K. Yokogawa, Research and Development Division, Dainippon Pharmaceutical Co. (Suita, Osaka).

2. *Cell wall lytic or peptidoglycan-degrading enzymes*

The modes of action and substrate specificities of the enzymes used in this study are summarized in Table 1. These properties together with the methods used for isolation and purification of enzymes, were reported previously; L–11 enzyme (Kato et al., 1962; Kato and Strominger, 1968; Kato et al., 1968; Matsuda, Kotani and Kato, 1968b; Suginaka et al., 1970; Hirata, 1970; Hamada et al., 1971; Hirachi et al., 1971), L–3 enzyme (Mori et al., 1960; Mori and Kotani, 1962; Kato, Strominger and Kotani, 1968; Matsuda, Kotani and Kato,

TABLE 1. *Linkages in the cell wall peptidoglycans hydrolyzed by the Flavobacterium L-11 enzyme (or Kyowa lytic # 2 enzyme), Streptomyces L-3 enzyme, Mutanolysin, Chalarospis B enzyme and egg white lysozyme used in the present study*

Enzyme	Linkage hydrolyzed
Flavobacterium L-11 enzyme (or Kyowa lytic # 2 enzyme)	-D-Ala-Gly- -D-Ala-L-Ala- -Gly-Gly- (-D-Ala-*meso*-Dpm-) -MurNAc-L-Ala-
Streptomyces L-3 enzyme	-D-Ala-*meso*-Dpm- (-D-Ala-ε-L-Lys-) -MurNAc-L-Ala-
Streptomyces globisporus enzyme (Mutanolysin)	-MurNAc-β-1, 4-GlcNAc- -Mur *N*, 6-*O*-diAc-β-1, 4-GlcNAc- (-MurNAc-L-Ala-) (endopeptidase)
Chalaropsis B enzyme	-MurNAc-β-1, 4-GlcNAc- -Mur *N*, 6-*O*-diAc-β-1, 4-GlcNAc-
Egg white lysozyme	-MurNAc-β-1, 4-GlcNAc-

1968a; Matsuda et al., 1968; Katayama, 1973), Mutanolysin (Yokogawa, Kawata and Yoshimura, 1972, 1973; Yokogawa et al., 1974), *Chalaropsis* B enzyme (Hash, 1963; Hash and Rothlauf, 1967; Mitchell and Hash, 1969; Shin and Hash, 1971). The Kyowa lytic #2 enzyme, which is essentially the same muralytic enzyme as the L-11 enzyme, was generously supplied by Kyowa Fermentation Industry (Tokyo), and purified as described in a previous paper on the L-11 enzyme (Hamada et al., 1971). The Mutanolysin and *Chalaropsis* B enzyme were gifts from Drs. K. Yokogawa and S. Kawata, and Dr. P. A. Miller (Lederle Laboratories, American Cyanamide Co., New York, U.S.A.), respectively.

3. *Preparation of water-soluble higher molecular weight components (HMWC's) from representative adjuvant-active cell walls*

Staphylococcus aureus (Copenhagen): A higher molecular weight fraction obtained from the L-11 enzyme digest by gel filtration as described previously (Kato et al., 1968) was applied to an ECTEOLA-cellulose column. The column was developed first with deionized water and then with a linear gradient of increasing molarity of LiCl, pH 5.0. Fractions, ECTEOLA/LiCl-1 and -2 (see Table 4), eluted with LiCl were examined for immunoadjuvant activity.

Streptococcus pyogenes (group A, type 6; S43/100): A preparation of cell walls was digested at 37 C with the L-11 enzyme (0.1 unit/mg cell walls) in 0.005 M phosphate buffer, pH 8.0 for 96 hr, liberating 0.67 mole of free amino-groups/mole of total glutamic acid residue in the substrate cell walls (This method of expression of the liberation of terminal groups is used throughout). The digest was centrifuged and the insoluble material was again treated with the L-11 enzyme. Materials solubilized by the first and second digestions were combined, and submitted to gel filtration on a Sephadex G-50 column connected in series with a G-25 column to isolate a higher molecular weight component (39% of the starting material). Another aliquot of the same cell wall preparation was incubated with partially purified Mutanolysin (one unit/mg of walls) in 0.005 M phosphate buffer, pH 8.0 at 37 C. After 96 hr 80% reduction in the optical density and release of 1.8 moles of free amino-groups and 1.1 moles of reducing groups were observed. In a similar way, a higher molecular weight fraction was isolated in 51% yield from the Mutanolysin digest by gel filtration.

Part of the HMWC from the L-11 enzyme digest was further treated at 37 C with purified Mutanolysin (50 units/mg HMWC) in 0.1 M acetate buffer, pH 5.4 for 96 hr. During this incubation, 0.8 mole of reducing groups and 0.3 mole of amino-groups were liberated. The digest was submitted to gel filtration on the same Sephadex columns used in filtration of the L-11 enzyme digest described above to separate an L-11/Mutanolysin HMWC (in 56% yield).

Str. salivarius (IFO 3350), *Str. faecalis* (IFO 12580) and *Str. mutans* (BHT): Higher molecular weight components from these species were gifts from Drs. S. Kawata and K. Yokogawa.

Lactobacillus plantarum (ATCC 8014): The *Chalaropsis* B enzyme was used for solubization of the cell walls. Liberation of 1.4 moles of reducing groups without any significant release of free amino-groups was observed on incubation for 8 hr. The *Chalaropsis* enzyme digest was chromatographed on an ECTEOLA-cellulose column. The neutral or basic fraction eluted from the column with water was applied to three successive Sephadex columns of G-50 (fine), G-50 (coarse) and G-25 (coarse) gels. In this way a higher molecular weight fraction (an ECTEOLA/H_2O → Sephadex fraction) was obtained. After elution with water, the ECTEOLA

column was developed with a linear gradient of increasing molarity of LiCl, pH 5.0. The resulting acidic eluate was then submitted to chromatography on a DEAE-cellulose column. An ECTEOLA/LiCl → DEAE/NaCl fraction was eluted with a linear gradient of increasing NaCl concentration and examined for possible immunoadjuvancy. Another preparation of HMWC was obtained from the L-3 enzyme digest as described in a previous paper (Matsuda, Kotani and Kato, 1968a).

Bacillus megaterium (IFO 12068): An HMWC from this organism was kindly supplied by Drs. K. Yokogawa and S. Kawata.

Corynebacterium diphtheriae (Park-Williams No. 8): A delipidated cell wall preparation was solubilized by treatment with the *Chalaropsis* B enzyme (0.2 mg/100 mg) in 0.01 M acetate-buffer, pH 4.7 for 8 hr at 37 C, liberating 0.57 mole of reducing groups. The solubilized materials were applied on an ECTEOLA-cellulose column. A fraction containing a little arabinose, eluted with deionized water, was subjected to gel filtration on connected columns of Sephadex G-50 and G-25 gels. A fraction consisting of amino sugars and amino acids with virtually no pentose was separated and assayed for adjuvant activity.

Actinomyces viscosus (ATCC 15987): A cell wall specimen was digested with partially purified Mutanolysin (5 whole cell lytic units/mg substrate) in 0.01 M Tris-HCl buffer, pH 7.0 at 37 C. The cell walls were almost completely solubilized liberating 0.64 mole of reducing groups. The soluble materials were lyophilized without further fractionation and assayed for adjuvant activity.

Mycobacterium smegmatis: A higher molecular weight component was prepared from the cell walls digested with the Kyowa lytic #2 enzyme or egg white lysozyme and kindly given by Dr. O. Kohashi, Research Institute for Diseases of the Chest, Faculty of Medicine, Kyushu University.

4. *Preparation of cytoplasmic membrane fractions*

A cytoplasmic membrane fraction of *S. aureus* (FDA 209P) was prepared by treatment of cells with Lysostaphin (Browder et al., 1965: Tipper and Strominger, 1966; Schindler and Schuhardt, 1964, 1965) (Schwarz/Mann, New York, U.S.A.) in 0.03 M Tris-HCl buffer, pH 7.5 at 37 C to remove cell wall peptidoglycans, treatment of the resulting ghost cells with deoxyribonuclease and repeated washing (Hirachi et al., 1974). A membrane fraction of *Micrococcus lysodeikticus* (NCTC 2665) was isolated by osmotic shock of protoplasts, prepared using crystalline egg white lysozyme, and treatment of the ruptured cells with nucleases (Hirachi, 1973). The latter membane fraction was a generous gift from Dr. Y. Hirachi of this laboratory. A fraction derived from the cytoplasmic membranes of BCG was prepared from 6 days-old cells grown homogeneously by shaking culture in Tween 80-casaminoacid medium as described by Fukui et al. (1965), by disruption of the cells in a Ribi cell fractionator at 20,000 psi and then differential centrifugation. The fraction not precipitated by centrifugation at 40,000 *g* for one hr but precipitated by centrifugation at 100,000 *g* for one hr (Kotani et al., 1973) was assayed for adjuvant activity. The cytoplasmic membranes from *Str. mutans* (FA-1) were isolated using purified Mutanolysin and kindly supplied by Drs. S. Kawata and K. Yokogawa.

All the cytoplasmic membrane fractions described above were shown to be virtually uncontaminated with cell wall components by chemical and immunological methods.

5. *Assay of immunoadjuvant activities*

Groups of 5 female albino guinea-pigs (200-300 g) were injected in the left hind foot-pad with 0.2 ml of a water-in-oil emulsion containing 1 mg of crystalline ovalbumin (Grade V, Sigma) as antigen and 100 or 200 μg of either cell walls or their enzymatic digests (water-soluble, higher molecular weight components) or cytoplasmic membrane fractions. The water-in-oil emulsion consisted of Drakeol 6VR (Pennsylvania Refining Co., Penn., U.S.A.), Arlacel A (Atlas Chemical Industries Inc., Del., U.S.A.) and physiological saline in a ratio of 4:1:5, by volume. Control guinea-pigs were injected with water-in-oil emulsion of similar composition containing the antigen, but no test preparation (Freund's incomplete-type adjuvant, abbreviated as FICA).

To test for the induction of delayed-type hypersensitivity to ovalbumin, 3 weeks after the injection the guinea-pigs were given an intracorneal injection of ovalbumin solution (20 mg/ml saline) to make a transient disc of opacity of approximately 5 mm in diameter. The eyes were examined after 24 and 48 hr and the extent and degree of corneal opacity were recorded (Holley, 1935; Rich and Follis, 1940; Raffel et al., 1949; White, Coons and Connolly, 1955; Kotani et al., 1963a; Kotani et al., 1963b;

Stewart-Tull and White, 1967). The reactions were graded from 3.0, for a strong reaction where the whole cornea was thickened, opaque and greyish-white, to 1.0, where slight opacity was observed. When no visible difference from the uninjected eye was detected the reaction was graded as 0. A few days later, the early and delayed skin responses were determined using an ovalbumin solution (0.1 mg/0.1 ml slaine). One week to 10 days after the corneal test the animals were bled by cardiac puncture. The serum sepcimens were stored at −20 C. The levels of antiovalbumin antibody nitrogen in the sera were measured quantitatively using a spectrophotometric technique (Stewart-Tull and White, 1967). The presence of IgG_2-immunoglobulin specific for ovalbumin was revealed by agar gel immunoelectrophoresis (White, Jenkins and Wilkinson, 1963). The intensity of IgG_2 arcs was graded arbitrarily from 0 to 3.0.

6. *Chemical analyses*

Essentially the same methods as those described in a previous paper (Iwata et al., 1972) were followed for determination of component amino sugars or amino acids and amino-terminal and carboxyl-terminal amino acids, and for estimation of the chain lengths of the glycan portions of test peptidoglycan subunits.

Liberation of reducing groups (as glucosamine) and free amino-groups (as glutamic acid) were determined by the Park and Johnson ferricyanide method (1949) and the method described by Ghuysen, Tipper and Strominger (1966), respectively.

Pentose and hexose were measured quantitatively by the orcinol and anthrone methods, respectively as described by Ashwell (1957). Methylpentose as rhamnose was assayed by the cystein-sulfuric acid procedure of Dische and Shettles (1948) without prior hydrolysis. Total hexosamines (as glucosamine), total and organic phosphorus and ammonia were determined by the methods of Roseman and Daffner (1956) modified by Ghuysen, Tipper and Strominger (1966), Lowry et al. (1954) and Fawcett and Scott (1960), respectively.

RESULTS

1. *Comparison of the immunoadjuvant activities of cell walls from various gram-positive bacteria*

Cell wall preparations obtained from 21 species of gram-positive bacteria were examined for immunoadjuvant activity. The results are summarized in Table 2. With only two exceptions (those of *M. lysodeikticus* NCTC 2665 and *Staphylococcus epidermidis* ATCC

TABLE 2. *Summary of assays for the immunoadjuvant activities of cell wall preparations obtained from various species of gram-positive bacteria*

No. of experimental group	Source of cell walls	Corneal response (48 hr) Mean (Range)	Antibody level (Ratio)[a] Mean±S.E.[d]	IgG_2 Mean (Range)
Test group				
3	*M. lysodeikticus* (NCTC 2665)	0	1.2±0.43	0.4 (0 -2.0)
4	*S. aureus* (FDA 209P)	1.6 (1.0-2.0)	3.1±0.35**	1.8 (0.5-3.0)
12	*S. aureus* (Copenhagen)	3.0	ND[c]	ND
3	*S. epidermidis* (ATCC 155)	0	1.4±0.10*	0
6	*S. epidermidis* (ATCC 155)[b]	0	1.1±0.17	0.6 (0.5-1.0)
4	*G. tetragena* (ATCC 15292)	1.8 (0 -3.0)	3.5±0.87*	0.9 (0 -3.0)
5	*Sar. lutea* (IFO 3232)	2.5 (1.0-3.0)	2.0±0.63	1.8 (0.5-3.0)
1	*Str. pyogenes* (S43/100)	2.6 (2.0-3.0)	2.2±0.36*	1.7 (0.5-3.0)
12	*Str. pyogenes* (S.F. 42)	3.0	ND	ND
4	*Str. salivarius* (HHT)	2.2 (1.0-3.0)	1.0±0.21	2.0 (0 -3.0)
7	*Str. salivarius* (HHT)	1.9 (0 -3.0)	3.4±0.74*	1.5 (0 -3.0)
11	*Str. salivarius* (IFO 3350)	1.6 (0 -2.5)	1.6±0.34	ND

Continued . . .

TABLE 2. *Continued.*

No. of experimental group	Source of cell walls	Corneal response (48 hr) Mean (Range)	Antibody level (Ratio)[a] Mean±S.E.[d]	IgG_2 Mean (Range)
8	*Str. salivarius* (IFO 3350)[b]	3.0	6.3±0.61**	3.0
4	*Str. faecalis* (IFO 12580)	3.0	3.8±0.42**	3.0
4	*Str. faecium* (IFO 3181)	1.4 (1.0-3.0)	3.2±0.69*	0.4 (0 -2.0)
6	*Str. bovis* (IFO 12058)	2.0 (2.0-2.0)	3.4±0.54**	2.5 (1.0-3.0)
4	*Str. lactis* (IFO 12546)	2.6 (2.0-3.0)	3.8±0.76**	2.8 (2.5-3.0)
4	*Str. thermophilus* (IFO 3535)	3.0	3.9±1.1*	2.0 (0.5-3.0)
3	*Str. mutans* (BHT)	3.0	4.5±0.37**	3.0
11	*Str. mutans* (KIR)	1.6 (0 -3.0)	2.1±0.50*	ND
12	*Streptococcus* sp. (CHT)	2.6 (2.0-3.0)	ND	ND
3	*L. plantarum* (ATCC 8014)	2.7 (2.0-3.0)	2.8±0.27**	1.3 (0.5-2.0)
2	*B. megaterium* (KM)	1.3 (1.0-2.0)	3.3±1.0	2.0 (2.0-2.0)
2	*Cl. botulinum* (190L)	1.9 (1.0-3.0)	2.2±0.27	0.6 (0 -1.0)
10	*Myc. bovis*, BCG (Takeo)[b]	2.5 (2.0-3.0)	6.0±0.84**	2.6 (2.0-3.0)
3	*N. asteroides* (131)	2.4 (1.5-3.0)	2.6±0.28**	2.5 (0.5-3.0)
9	*Stm. lavendurae* (IAM 0023)	1.8 (1.0-3.0)	2.8±1.0*	0.8 (0 -3.0)
9	*Stm. fradiae* (IAM 0093)	1.6 (1.0-2.0)	2.4±1.2	1.2 (0 -3.0)
Control group				
1	FICA-type control	0.6 (0 -1.0)	[219±52][c]	0
2	,, ,,	0	[45±23]	0
3	,, ,,	0.4 (0 -1.0)	[67± 8]	0.3 (0 -1.0)
4	,, ,,	0	[106± 7]	0.6 (0 -2.0)
5	,, ,,	0.2 (0 -1.0)	[212±55]	0·8 (0 -2.0)
6	,, ,,	0	[167±16]	0.4 (0 -1.0)
7	,, ,,	0	[82±31]	ND
8	,, ,,	0	[99±50]	ND
9	,, ,,	0.5 (0 -2.0)	[56±16]	ND
10	,, ,,	0.2 (0 -1.0)	[145±41]	0
11	,, ,,	0	[149±26]	0
12	,, ,,	0	ND	ND

[a] Ratio of antibody nitrogen (μg/ml serum specimen) in the test group to that in the respective control group.
[b] The amount of test specimens injected in adjuvant mixture was 200 μg/guinea-pig. In other cases 100 μg were injected.
[c] μg Antibody nitrogen/ml serum specimen.
[d] The difference between values in the test and respective control groups was significant at a level of 5% (*) or 1% (**), by the "Student" t-test.
[e] Not determined.

155) the cell walls from all the test bacteria were shown to be definitely active in both stimulation of increased circulating antibody levels and in induction of delayed-type hypersentitivity to crystalline ovalbumin in guinea-pigs. It should be mentioned that the serum antibody levels in the test groups in this and the following tables are expressed as ratios of the antibody nitrogen (μg/ml serum specimen) in the test group to that of the respective con-

TABLE 3. *Test of immunoadjuvant activities of cytoplasmic membrane fractions from several bacterial species*

No. of experimental group	Source of membrane fraction from[a]	Corneal reponse (48 hr) Mean (Range)	Antibody level (Ratio)[b] Mean±S.E.	IgG_2 Mean (Range)
13	*M. lysodeikticus* (NCTC 2665)	0	1.8 ±0.49	0.1 (0 -0.5)
13	*S. aureus* (FDA 209P)	0.2 (0 -1.0)	0.87±0.20	0
13	*Str. mutans* (FA-1)	0	0.52±0.30	0.1 (0 -0.5)
13	*Myc. bovis*, BCG (Takeo)	0	1.0 ±0.37	0
13	FICA-type control	0	[104±10][c]	0

[a] The amount of test specimen injected in adjuvant mixtures was 200 μg/guinea-pig.
[b] Ratio of antibody nitrogen (μg/ml serum specimen) in the test group to that in the respective control group.
[c] μg Antibody nitrogen/ml serum specimen.

trol group, because the actual serum antibody levels in the FICA control groups were found to fluctuate considerably, and so the relative ratios seemed to be more convenient for comparison of serum antibody levels in the test groups in different sets of experiments.

The above results proved that the immunological adjuvant activity of bacterial cell walls was by no means a generic character confined to the genus *Mycobacterium* and phylogenetically related bacteria, as was believed generally until recently, but was a property common to many gram-positive bacteria, although the immunoadjuvant potency of the cell walls of each species seemed to be variable.

As a control study, the immunoadjuvant activities of the cytoplasmic membrane fractions prepared from a few bacterial species, including BCG, were examined. None of the test membrane preparations had significant adjuvant activity, as shown in Table 3. This supports the view that immunoadjuvancy is a characteristic of the cell walls.

2. *Solubilization of an adjuvant-active component from bacterial cell walls using peptidoglycan-degrading enzymes*

The immunoadjuvant activities of the water-soluble, higher molecular weight components obtained by gel filtration and/or ion-exchange chromatography of cell wall digests of several bacterial species, prepared by treatment with an appropriate cell wall lytic enzyme were examined. Table 4 shows the results of adjuvant assays together with the postulated chemical characteristics of some of these HMWC's. Those from the enzymatic digests of the cell walls of *S. aureus*, group A *Str. pyogenes*, *Str. salivarius*, *Str. faecalis*, *Str. mutans*, *L. plantarum* and *B. megaterium* had definite adjuvant activity like the HMWC's derived from the cell walls of *Myc. smegmatis* and related species, such as *C. diphtheriae* and *A. viscosus*, in inducing delayed-type hypersensitivity and in stimulating increased circulating antibody levels to ovalbumin, when administered as water-in-oil emulsions to guinea-pigs. The postulated chemical structures of these HMWC's were based on the modes of hydrolytic action of the enzymes used in their preparation and the results of chemical analyses of the specimens. Analytical data are given in Table 5. Some HMWC's could not be analyzed because too little material was available. References are also given to available information on the chemical characteristics of the walls of the test organisms (e.g. *S. aureus*/Kato and Strominger, 1968; Kato et al., 1968; *Str. pyogenes*/Hamada, Kotani and Kato, 1968; *Str. mutans*/Inoue et al., 1971; *L. plantarum*/Matsuda, Kotani and Kato, 1968a, 1968b; *C. diphtheriae*/Kato, Strominger and Kotani, 1968; *Mycobacterium*/Kotani et al., 1970). As in intact cells, the type of diamino acid residue

TABLE 4. *Induction of delayed-type hypersensitivity ana stimulation of increased serum antibody from bacterial cell walls by peptidoglycan-degrading enzymes*

No. of experimental group	Higher molecular weight component	Enzyme used	Corneal response (48 hr) Mean (Range)
	S. aureus (Copenhagen)		
1	Unfractionated HMWC	L-11	3.0
3	ECTEOLA/LiCl-1	L-11	3.0
2	ECTEOLA/LiCl-2	L-11	2.6 (2.0-3.0)
	Str. pyogenes (S43/100)		
1	Unfractionated HMWC	L-11	3.0
1	Unfractionated HMWC	Mutanolysin	2.3 (1.0-3.0)
3	Unfractionated HMWC	L-11/Mutanolysin	2.0 (1.0-3.0)
	Str. salivarius (IFO 3350)		
14	Unfractionated HMWC	Mutanolysin	2.8 (2.5-3.0)
	Str. faecalis (IFO 12580)		
14	Unfractionated HMWC	Mutanolysin	2.7 (2.0-3.0)
	Str. mutans (BHT)		
3	Unfractionated HMWC	Mutanolysin	2.8 (2.0-3.0)
	L. plantarum (ATCC 8014)		
2	Unfractionated HMWC	L-3	2.1 (1.0-3.0)
2	ECTEOLA/H_2O → Sephadex	Chala	2.3 (1.5-2.5)
2	ECTEOLA/LiCl → DEAE/NaCl	Chala	2.6 (1.0-3.0)
	B. megaterium (IFO 12068)		
14	Unfractionated HMWC	Mutanolysin	2.6 (2.0-3.0)
	C. diphtheriae (Park-Williams No. 8)		
2	ECTEOLA/H_2O → Sephadex[e]	Chala	3.0
	Myc. smegmatis		
3	Unfractionated HMWC	Lysozyme	3.0
3	Unfractionated HMWC	Kyowa	3.0
	A. viscosus (ATCC 15987)		
3	Unfractionated lyzate	Mutanolysin	2.9 (2.5-3.0)
5	L-11 enzyme control	—	0.6 (0 -2.0)
5	Mutanolysin control	—	0.2 (0 -1.0)
14	FICA-type control[f]	—	0

[a] Ratio of antibody nitrogen (μg/ml serum specimen) in the test group to that in the respective control group.

[b] Rham : rhamnose, GlcNAc : *N*-acetylglucosamine, Gal : galactose, Glc : glucose, Ara : arabinose, Teichoic acid[c] : *N*-acetylglucosaminyl ribitol teichoic acid, Teichoic acid[d] : glucosyl ribitol teichoic acid.

[e] The amount of test specimen injected in adjuvant mixture was 200 μg/guinea-pig. In other cases it was 100 μg.

in the peptide protion [i.e., L-lysine or *meso*-2,6-diaminopimelic acid (Dpm)] does not influence the existence of immunoadjuvant activity in the HMWC.

It may be added here that the preparations of the L-11 enzyme and Mutanolysin used in the present study were shown in a control experiment to have no significant immuno-

levels to ovalbumin in guinea-pigs by water-soluble higher molecular weight components liberated

Antibody level (Ratio)[a] Mean±S.E.[h]	IgG_2 Mean (Range)	Postulated chemical structure[b]
5.0±0.61**	3.0	
6.4±0.43**	2.4 (2.0–3.0)	Glycan-oligopeptide
7.0±0.74**	2.0 (2.0–2.0)	Teichoic acid[c]-glycan-oligopeptide
5.0±0.17**	3.0	Rham, GlcNAc polymer-glycan-oligopeptide
ND	ND[i]	Rham, GlcNAc polymer-oligosaccharide-peptide
ND	1.4 (0 –2.0)	Rham, GlcNAc polymer-oligosaccharide-oligopeptide
2.2±0.39	1.6 (0.5–3.0)	
3.4±0.39**	2.5 (2.0–3.0)	
2.9±0.65	2.3 (0 –3.0)	Rham, Gal polymer-oligosaccharide-peptide
2.7±0.73*	ND	
5.2±0.31**	1.8 (1.0–2.0)	Rham, Glc polymer-oligosaccharide-peptide
4.3±0.88*	2.1 (0.5–3.0)	Teichoic acid[d]-oligosaccharide-peptide
2.9±0.61*	1.8 (0 –3.0)	
5.0±0.31**	2.4 (2.0–3.0)	Disaccharide-tetrapeptide-tripeptide-disaccharide
2.5±0.42**	ND	Ara, Gal polymer-oligosaccharide-peptide
3.7±0.34**	ND	Ara, Gal polymer-glycan-oligopeptide
2.0±0.21**	2.4 (1.5–3.0)	
1.2±0.37	0.5 (0 –2.0)	
1.0±0.17	0.3 (0 –1.0)	
[174±49][g]	0.1 (0 –0.5)	

[f] The FICA-type controls for experimental groups Nos. 1, 2, 3 and 5 were the same as in Table 2.
[g] μg Antibody nitrogen/ml serum specimen.
[h] The difference between values in the test and respective control groups was significant at a level of 5% (*) or 1% (**), by the "Student" t-test.
[i] Not determined.

adjuvant activities, when administered to guinea-pigs as water-in-oil emulsions with ovalbumin, even in much higher doses than those estimated to be present in the test HMWC's prepared using these enzymes. No control study was made on the *Chalaropsis* B enzyme or L–3 enzyme because with the former the amount of enzyme used relative to that

TABLE 5. *Analyses of amino acids, amino sugars and other components in the non-peptidoglycan portion ("Special structure") in the higher molecular weight components from the cell walls of gram-positive bacteria*[a]

Test specimen	GlcN[b]	Mur[a,b]	Ala	Glu	Lys [Dpm]	Gly	M6P[b]	Others[b]
S. aureus (Copenhagen)								
Unfractionated HMWC (L-11)	1.00	0.78	2.75	1.00	1.09	2.96	+	GlcN (1.82)
Str. pyogenes (S43/100)								
Unfractionated HMWC (L-11)	1.00	1.04	3.39	1.00	1.16		+	GlcN (2.13) Rham (4.91)
Unfractionated HMWC (Mutanolysin)	1.00	0.73	3.94	1.00	1.26		+	GlcN (4.85) Rham (8.40)
Unfractionated HMWC (L-11/Mutanolysin)	1.00	0.91	3.74	1.00	1.19		+	GlcN (6.36) Rham (9.25)
L. plantarum (ATCC 8014)								
ECTEOLA/H_2O → Sephadex (Chala)								
[1][c]	1.00	1.13	1.56	1.00	[1.03]			Hex (1.8) Rham (1.5) P_0 (0.37) Unhydroribitol (−)
[2]	1.17	1.00	1.48	1.00	[0.65]			
[3]	1.09	0.50	1.55	1.00	[1.02]			
ECTEOLA/LiCl → DEAE/NaCl (Chala)								
[1]	1.35	0.56	1.47	1.00	[0.88]			Hex (18) Rham (0.47) P_0 (0.37) Unhybroribitol (+)
[2]	1.16	ND[e]	1.51	1.00	[0.48]			
[3]	1.41	tr[f]	1.56	1.00	[0.93]			

[a] A factor of 1.47 was used to correct for destruction of muramic acid during acid hydrolysis.
[b] GlcN: Glucosamine, Mur: muramic acid, M6P: muramic acid-6-phosphate.
[c] [1]: Untreated, [2]: dinitrophenylated, [3]: reduced with $NaBH_4$.
[d] All data are expressed as moles/mole of glutamic acid residue.
[e] Not determined.
[f] Trace.

of cell walls was very small and with the latter the specimen employed was exhausted before the test could be made. The possible adjuvant activities of the Kyowa lytic #2 enzyme were not tested, because this enzyme was essentially the same as the L-11 enzyme.

3. *Relation of the corneal response, delayed skin response and IgG_2 type of antibody*

The relations between the corneal response and skin response and between the corneal response and IgG_2 type of anti-ovalbumin were analyzed using the data (averages for each group) presented in Tables 2 and 3 in this study and in Table 2 in the succeeding paper (Kotani et al., 1975). Figs. 1 and 2 show that there is a statistically significant correlation both between the strengths of the corneal response (after 48 hr) and the skin response (after 48 hr) and also between the former and the extent of production of an IgG_2 type of anti-ovalbumin antibody.

DISCUSSION

Immunological adjuvant activities (i.e. induction of delayed-type hypersensitivity and stimulation of humoral antibody to ovalbumin in guinea-pigs) have been shown to be common characteristics of the cell walls of many gram-positive bacteria. These characteristics are probably also associated with the cell walls of

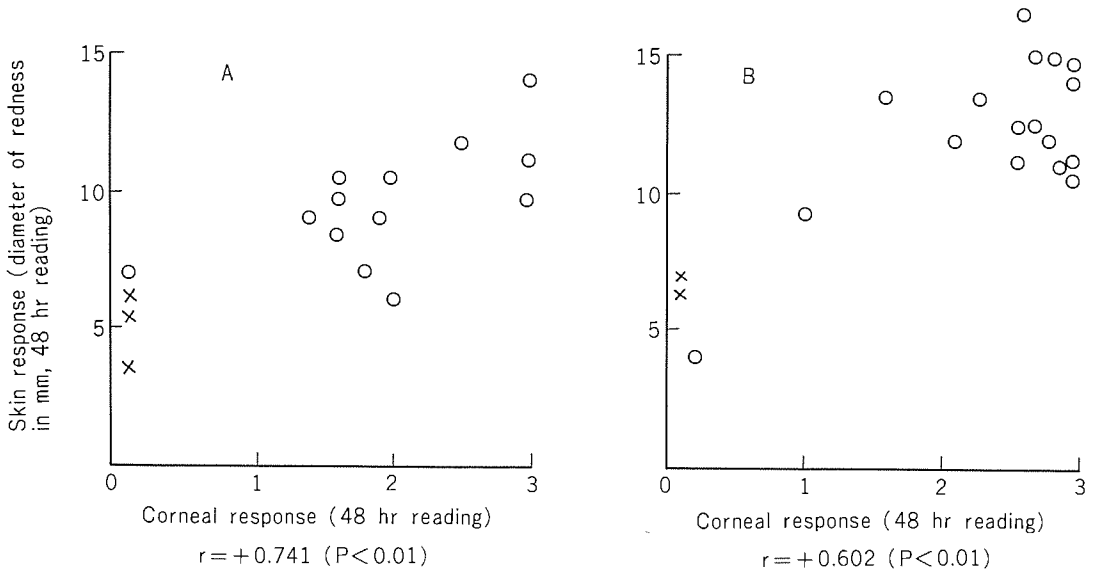

FIGURE 1. *Correlation between the strengths of the corneal response and delayed skin response.*
A: Cell wall preparations, B: Higher molecular weight components isolated from cell walls,
×: FICA-type controls.

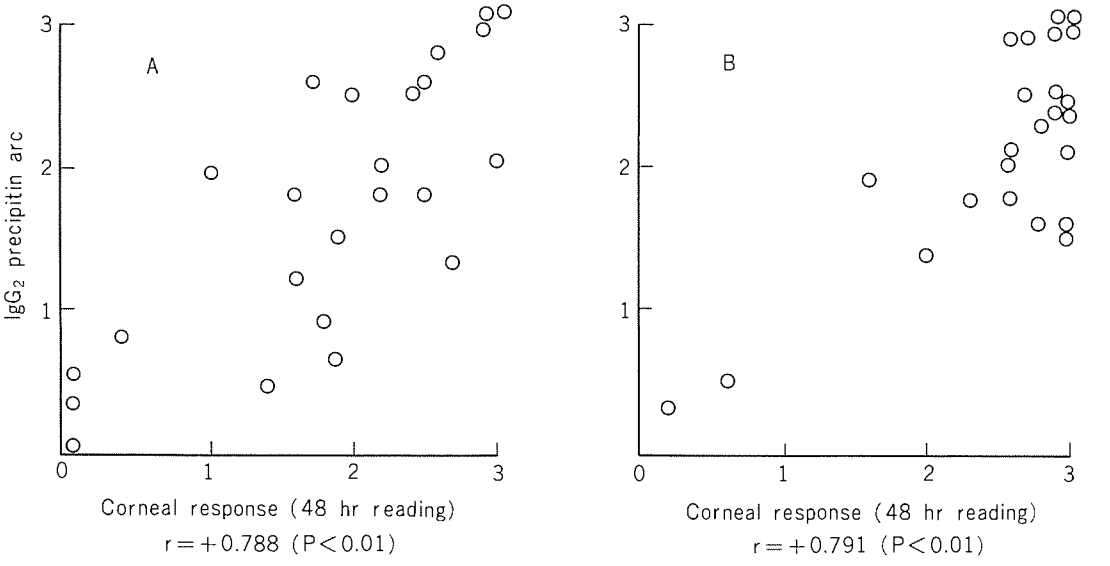

FIGURE 2. *Correlation between strengths of the corneal response and anti-ovalbumin IgG_2 precipitin arc.*
A: Cell wall preparations, B: Higher molecular weight components isolated from cell walls.

gram-negative bacteria. In the latter case, however, the participation of endotoxic lipopolysaccharide, an essential component of gram-negative bacterial cell walls, in the manifestation of adjuvant activities should be considered. It may be added here that while this

work was in progress adjuvant activities associated with the cell walls of a few species of gram-positive and gram-negative bacteria were reported, independently by other workers (Nguyen-Dang et al., 1973a, b; Nauciel et al., 1973, 1974).

The lack of adjuvant activity of *M. lysodeikticus* cell walls was not entirely unexpected, at least at this stage in the study, because the highly lysozyme-susceptible cell walls of this organism are probably rapidly disintegrated by the action of tissue lysozyme in guinea-pigs. However, the observation that *S. epidermidis* (ATCC 155) cell walls did not stimulate increased antibody production or development of a positive corneal response to ovalbumin was contrary to expectation. The cell walls of *S. epidermidis* used in this study are lysozyme-resistant and similar in their chemical characteristics to the walls of *S. aureus* which show definite adjuvant activity (Tipper and Berman, 1969; Tipper, 1969). In view of the fact that positive adjuvant activity of peptidoglycan of *S. epidermidis* was reported by Fleck et al. (1974) using azobenzenearsonate-*N*-acetyl-L-tyrosine, i.e. a quite different test antigen from that in the present study, further work is required on the lack of activity of the cell walls of both *M. lysodeikticus* and *S. epidermidis*, especially of the latter.

In studies on enzymatic solubilization of immunoadjuvant-active components from the cell walls, solubilized peptidoglycans lacking a non-peptidoglycan moiety (a glycan-oligopeptide fraction from *S. aureus* walls and a disaccharide-tetrapeptide-tripeptide-disaccharide from *C. diphtheriae* walls) exhibited definite adjuvant activities. This indicates that the non-peptidoglycan portion of the cell walls is not essential for manifestation of immunoadjuvancy, as judged by stimulation of either humoral or cellular immune responses, when administered to guinea-pigs as a water-in-oil emulsion with ovalbumin as antigen. However, the possibility that the efficiency of immunoadjuvancy of an adjuvant-active water-soluble peptidoglycan derivative can be enhanced by the "coexistence" of some non-peptidoglycan components remains to be elucidated.

White, Jenkins and Wilkinson (1963) and Benarcerraf et al. (1963) reported that two precipitin arcs are seen on immunoelectrophoretic analyses of sera taken from guinea-pigs three weeks after injection of a adjuvant mixture containing peptidoglycolipid from *Mycobacterium tuberculosis* and protein antigen. Later, Askonas, White and Wilkinson (1965) demonstrated that the peptidoglycolipid from tubercle bacilli altered the immunological response so that an IgG_2-type of antibody was synthesized. A statistically significant relationship between the production of IgG_2-type antibody and delayed hypersensitivity was shown by Stewart-Tull (1966) and Wilkinson (1966). In the present study further evidence for this relationship was obtained by the correlation between the strength of the corneal response and the extent of production of IgG_2-type anti-ovalbumin antibody in guinea-pigs which had been immunized with water-in-oil emulsions consisting of ovalbumin as antigen and cell walls or their water-soluble fractions prepared from various gram-positive bacteria.

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